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CD16$^+$ monocytes in breast cancer patients: expanded by monocyte chemoattractant protein-1 and may be useful for early diagnosis

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Summary

Human peripheral blood monocytes are a heterogeneous population, including CD14$^+$CD16$^-$ 'classical' monocytes and CD14$^+$CD16$^+$ 'proinflammatory' monocytes. CD16$^+$ monocytes are expanded in various inflammatory conditions. However, little is known about the CD14$^+$CD16$^+$ monocytes in patients with breast cancer. We detected CD14$^+$CD16$^+$ monocytes in 96 patients with breast cancer and 54 control subjects using flow cytometry. Receiver-operating characteristic (ROC) curve analysis was used to determine the feasibility of CD14$^+$CD16$^+$ monocytes as an indicator for diagnosis of breast cancer. We found that the frequency of CD14$^+$CD16$^+$ monocytes showed a significantly greater increase in breast cancer patients than in controls (16·96% *versus* 10·84%, $P < 0·0001$). The area under the ROC curve for CD14$^+$CD16$^+$ monocytes was 0·805 [95% confidence interval (95% CI): 0·714–0·877, $P = 0·0001$]. Furthermore, the levels of CD16$^+$ monocytes were significantly negatively associated with the tumour size and pathological staging. *In vitro*, we showed that CD14$^+$CD16$^+$ monocytes were expanded significantly when the purified CD14$^+$ monocytes were exposed to Michigan Cancer Foundation (MCF)-7 cells-conditioned medium (MCF-CM) or, separately, to monocyte chemotactic protein 1 (MCP-1). Neutralizing antibodies against MCP-1 inhibited the expansion of CD14$^+$CD16$^+$ monocytes by MCF-CM. Collectively, our findings indicated that MCP-1 can expand CD14$^+$CD16$^+$ monocytes in patients with breast cancer. Furthermore, the CD14$^+$CD16$^+$ monocyte may be a useful indicator in early diagnosis of breast cancer.

Keywords: breast cancer, CD14$^+$CD16$^+$ monocytes, heterogeneity, early diagnosis, MCP-1

Introduction

Breast cancer is by far the most frequent cancer in women (23% of all cancers), with an estimated 1·15 million new cases yearly [1]. Despite the improved prognosis for breast cancer resulting from radical surgery and the development of adjuvant therapy, early diagnosis of breast cancer still remains a challenge [2]. It has been proved that an important function of the immune system is to search for and eliminate neoplastic cells [3]. Monocytes can differentiate into dendritic cells (DCs) and macrophages, and they can be involved in the anti-tumour response of the host [4]. The CD16 (FcγRIII) molecule was believed initially to be restricted to a subset of mature macrophages. However, it has also been found that a subset of circulating monocytes co-express CD14 and CD16. These CD14$^+$CD16$^+$ monocytes account for about 10% of all monocytes in healthy people, and their function is still unclear [5]. A few studies have demonstrated that CD14$^+$CD16$^+$ monocytes, also called proinflammatory monocytes, are characterized by the capacity to produce the proinflammatory cytokine tumour necrosis factor (TNF)-α [6]. Moreover, they produce little of the anti-inflammatory cytokine interleukin (IL)-10 in comparison to the main subpopulation of 'classical' CD14$^+$CD16$^-$ monocytes [7]. CD14$^+$CD16$^+$ monocytes share common features with DCs. They show a higher potential to differentiate DCs than regular CD14$^+$CD16$^-$ monocytes in a model of transendothelial trafficking [8]. CD14$^+$CD16$^+$ monocyte-derived DCs (CD16$^+$ mDCs) seem to promote T helper type 2 (Th2) responses preferentially by comparison with CD14$^+$CD16$^-$ mDCs [9,10]. CD14$^+$CD16$^+$ monocytes are elevated in various inflammatory diseases such as sepsis, asthma and

coronary artery disease [11–13]. However, to date the mechanism by which $CD14^+CD16^+$ monocytes increase remains unclear.

Tumours can mimic key features of lymph nodes and create a tolerant microenvironment, allowing tumours to be better able to escape from immunological attack. Chemokines are a superfamily of low molecular weight cytokines that selectively attract and activate different cell types [14]. Many pathophysiological conditions require the participation of chemokines, including malignant tumours. Chemokines play two contradictory roles in tumour immunity activity: they may enhance innate or specific host anti-tumour immunity, while they may also favour tumour growth and metastasis [15]. Monocyte chemoattractant protein-1 (MCP-1) is one of the key chemokines produced by immune cells, and is over-expressed in breast tumour cells and some other tumour cells [16,17]. MCP-1 can shift the balance between host anti-tumour immunity and tumour tolerance by increasing the presence of harmful tumour-associated macrophages (TAM) and by inhibiting anti-tumour T cell activities [17,18]. However, it remains unclear whether these $CD14^+CD16^+$ monocytes are associated with varying and different levels of risk of cancer. It also remains unclear whether the level of MCP-1 in co-cultured tumour cells interferes with monocyte heterogeneity.

In this paper, we report that the frequency of $CD14^+CD16^+$ monocytes is increased significantly in the peripheral circulation of breast cancer patients. Receiver-operating characteristic (ROC) curve analysis found that the frequency of $CD14^+CD16^+$ monocytes may be a useful indicator in the early diagnosis of breast cancer. Moreover, evidence has been gathered *in vitro* that the chemokine of MCP-1 can contribute to the expansion of $CD14^+CD16^+$ monocytes in the tumour microenvironment of breast cancer.

Materials and methods

Patients

Blood specimens were obtained from 96 patients with malignant breast cancer. Prior to participation in this study none of these patients had received any treatment, such as operation, chemotherapy, radiotherapy or immunotherapy, and none of these patients were suffering from any co-existing diseases that may cause increased levels of $CD14^+CD16^+$ monocytes such as rheumatoid arthritis (RA), atherosclerosis, haemodialysis, Crohn's disease, asthma, sepsis, human immunodeficiency virus (HIV) infection and other infectious diseases. For each of these 96 patients, the diagnosis of cancer was based on a clinical manifestation and auxiliary examinations, and the diagnosis was confirmed by postoperative pathology. All patients were female and the pathology was invasive ductal carcinoma. The cancers were staged according to the tumour–node–metastasis (TNM) classification system of the American Joint Committee on Cancer (AJCC). Fifty-four sex- and age-matched healthy donors with no diseases as described above served as control subjects. The study protocol was approved by the Institutional Review Board of Shandong University, and all subjects gave informed consent.

Cell line culture and conditioned medium preparation

The human mammary gland adenocarcinoma cell line Michigan Cancer Foundation (MCF)-7 was purchased from the American Type Culture Collection (ATCC) (ATCC HTR-22, Manassas, VA, USA). This cell line was grown in an atmosphere of 95% air and 5% CO_2 at 37°C in RPMI-1640 medium (Hyclone, Beijing, China) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. The MCF-7 cells-conditioned medium (MCF-CM) was prepared as described previously [19]. In outline, cells were seeded at 2×10^6 cells/75 cm^2 and cultivated until 60–70% confluence was reached. The medium was replaced and the supernatants were harvested after 48 h of further incubation.

Monocyte isolation

The use of human peripheral blood monocytes from healthy donors was approved by the Institutional Review Board of Shandong University. Peripheral blood mononuclear cells (PBMCs) were isolated from 50 ml heparinized peripheral blood using Ficoll-Paque Plus (Sigma-Aldrich, St Louis, MO, USA). $CD14^+$ cells from PBMCs were enriched with a bead-labelled anti-CD14 monoclonal antibody (mAb) (Miltenyi Biotec, Bergisch-Gladbach, Germany) using the magnetic antibody cell sorting (MACS) system (Miltenyi Biotec). The purity of $CD14^+$ monocytes was found routinely to be more than 90% as judged by flow cytometry analysis. The cell viability of each sample was >95% as determined by trypan blue dye.

Monocyte culture *in vitro*

$CD14^+$ monocytes (5×10^5/ml), purified from PBMCs, were cultured in complete RPMI-1640 (10% FBS, 2·05 mmol/l L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin) with either 25% (volume ratio of MCF-CM and total volume), 50% or 75% MCF-CM for 24 h in a 12-well plate. When required, recombinant human (rh)-MCP-1 (0·5 ng/ml, 10 ng/ml, 100 ng/ml), rh-TNF-α (50 ng/ml), rh-IL-2 (100 units/ml) and rh-osteopontin (OPN) (200 ng/ml) was added to the culture medium. In another experiment, monocytes were treated with 25% MCF-CM or rh-anti-MCP-1 mAb (5000 ng/ml). Each group of cells then was collected to determine the level of $CD14^+CD16^+$ monocytes by flow cytometry analysis. The supernatants were collected to examine cytokine secretion.

Flow cytometry analysis

Dual-colour immunofluorescence was performed using fluorescein isothiocyanate (FITC)-conjugated anti-CD14 monoclonal antibody (Becton-Dickinson, Mountain View, CA, USA) and phycoerythrin (PE)-conjugated anti-CD16 monoclonal antibody (Becton-Dickinson). Isotype controls were run in parallel with each of our experiments. Blood specimens were prepared for flow cytometry within 30 min after venipuncture and red blood cells were lysed with FACS lysing solution (Becton-Dickinson), then stained by direct immunofluorescence for FACS analysis. In the *in vitro* experiments the monocytes were collected after stimulation and washed with phosphate-buffered saline (PBS), then labelled with the corresponding mouse anti-human isotype-matched control antibodies or CD14 and CD16 monoclonal antibodies. The samples were acquired on a FACSCalibur flow cytometer (Becton-Dickinson) and the levels of different subsets of monocytes were calculated from the total $CD14^+$ monocyte population based on CD14 and CD16 expression using CellQuest software (Becton-Dickinson).

Enzyme-linked immunosorbent assay (ELISA)

The MCP-1 protein concentration in the culture supernatants, harvested from MCF-7 cells, was measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. In basic terms, a mouse mAb specific to MCP-1 was coated onto a microplate. Standards and samples were added to each well and incubated for 2 h at room temperature. After washing to remove unbound proteins, an enzyme-linked polyclonal antibody specific to MCP-1 was added to the wells and incubated for 2 h at room temperature. Following washing to remove the unbound antibody–enzyme reagent, the substrate solution was added to the wells and incubated for 30 min at room temperature in the dark. The optical density (OD) (450 nm) of each sample was determined using a microplate reader and the mean concentration of MCP-1 was calculated.

Statistical analysis

All the data analyses were performed using the software Statistical Package for Social Science (SPSS) version 13·0 for Windows (SPSS Inc., Chicago, IL, USA). The experimental data were expressed as mean ± standard deviation (s.d.). Group mean values were compared using one-way analysis of variance with the Newman–Keuls test. The feasibility of $CD14^+CD16^+$ monocytes as a clinical biomarker for breast cancer was assessed using ROC curve analysis. Intergroup comparisons of the clinical and pathological variables were analysed using a two-tailed χ^2 test for discrete variables. In each of the tests, statistically significant results were identified by $P < 0{\cdot}05$.

Results

Increased $CD14^+CD16^+$ monocyte frequency in breast cancer patients

We detected the different monocyte subsets in the peripheral blood of breast cancer patients ($n = 96$) and healthy donors ($n = 54$) by flow cytometry. Peripheral blood lymphocytes, monocytes and neutrophils were gated on the basis of forward- (FSC) and side-scatter (SSC) (Fig. 1a). Monocytes were confirmed by expression of the CD14 molecule (Fig. 1b). Most monocytes were $CD14^+CD16^-$ monocytes; they expressed CD14 intensely and did not express CD16. A minor population of monocytes co-expressing CD16 and

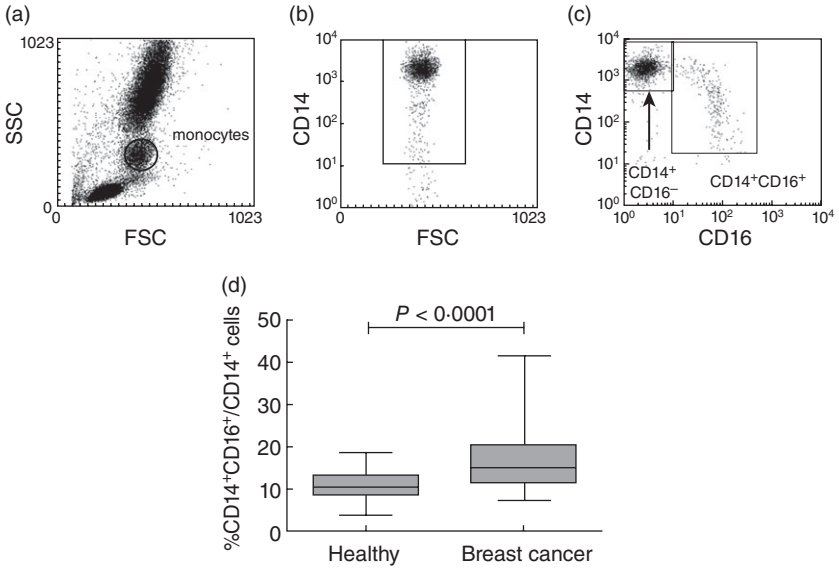

Fig. 1. Peripheral blood was stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 and phycoerythrin (PE)-conjugated anti-CD16 as described in Materials and methods. Monocytes were gated on the basis of the forward scatter and side-scatter dot plot (a) and identified using CD14 monoclonal antibodies (mAb) (b). According to the expression of CD14 and CD16, $CD14^+$ monocytes were subdivided into $CD14^+CD16^-$ and $CD14^+CD16^+$ subsets. Typical flow cytometry signals representative of a healthy subject (c) are shown. (d) The mean ± standard deviation frequency of $CD14^+CD16^+$ monocytes is shown for breast cancer patients ($n = 96$) and for healthy subjects ($n = 54$). $P < 0{\cdot}0001$.

CD14 were labelled as $CD14^+CD16^+$ monocytes (Fig. 1c). The results show that $CD14^+CD16^+$ monocytes exist in the peripheral blood of both healthy donors and patients with breast cancer. Furthermore, the frequency of $CD14^+CD16^+$ monocytes in patients with breast cancer were increased significantly by comparison with healthy controls (16·96 ± 7·7%, $n = 96$ *versus* 10·84 ± 3·6%, $n = 54$) ($P < 0·0001$) (Fig. 1d). The levels of $CD14^+CD16^-$ monocytes were not significantly different among cancer and healthy subjects (data not shown). We also prepared the single cell suspension of tumour tissues and then detected the $CD14^+CD16^+$ monocytes by flow cytometry. There existed a considerable proportion of $CD14^+CD16^+$ monocytes in tumour tissues (data not shown). These results demonstrate that $CD14^+CD16^+$ monocytes could be induced and expanded in breast cancer patients.

ROC curve analyses were carried out to assess the performance of $CD14^+CD16^+$ monocytes in the diagnosis of breast cancer

ROC curves were used to evaluate the performance of $CD14^+CD16^+$ monocytes in diagnosing breast cancer. The ROC curve analysis used breast cancers ($n = 96$) as the end-point for detection compared with healthy donors ($n = 54$). The data showed that the area under the ROC curve (AUC) was 0·805 ($P = 0·0001$) for the $CD14^+CD16^+$ monocytes assay (Fig. 2). The sensitivity and specificity of $CD14^+CD16^+$ monocytes were analysed using variable cut-off values.

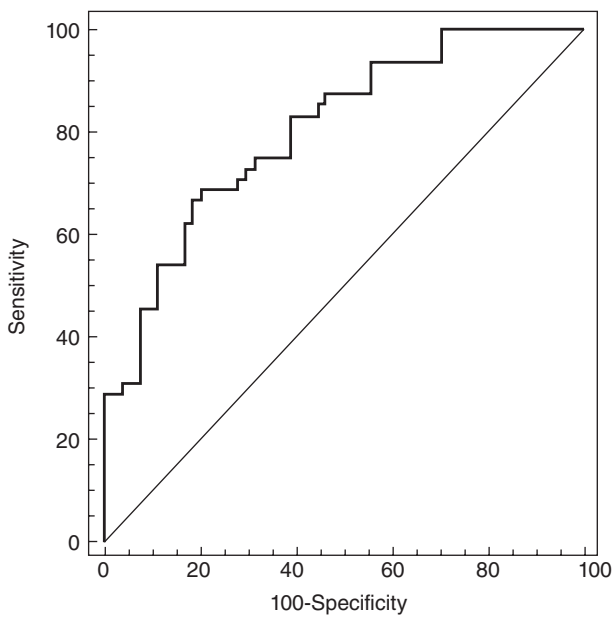

Fig. 2. Receiver-operating characteristic (ROC) curve analysis to assess the performance of $CD14^+CD16^+$ monocytes in the diagnosis of breast cancer. The area under the ROC curve was 80·5% [95% confidence interval (CI): 71·4–87·7%, $P = 0·0001$]. The cut-off value is 13·31, sensitivity 68·7% and specificity 79·6%.

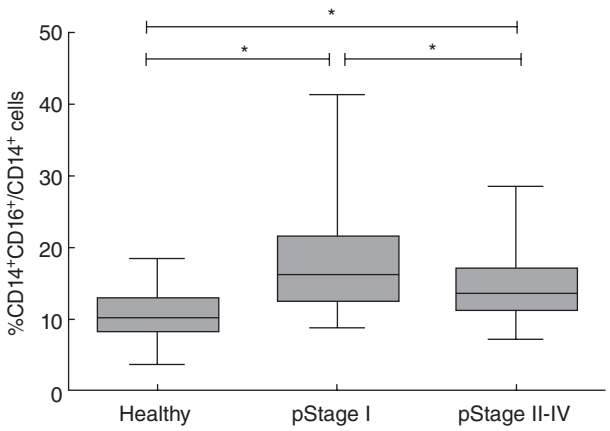

Fig. 3. The $CD14^+CD16^+$ monocytes were increased significantly in pstage I patients. Summary of analyses of the percentages of $CD14^+CD16^+$ monocytes in healthy subjects, pstage I and pstages II–IV breast cancer patients. $^*P < 0·05$.

Specifically, when the cut-point (13·31%) was used as the cut-off value, a sensitivity of 68·7% and specificity of 79·6% were achieved for the overall samples. This result demonstrates that the increased level of $CD14^+CD16^+$ monocytes in the peripheral blood may be a useful indicator in early diagnosis of breast cancer.

The high level of $CD14^+CD16^+$ monocytes is related to the early stage of breast cancer

Correlations between the level of $CD14^+CD16^+$ monocytes and patients' clinicopathological parameters such as age, TNM stage, menstruation and hormones were analysed by two-tailed χ^2 tests in this study (Table 1). We used the cut-off value of 13·31% in the ROC curve as the cut-point. As shown in Table 1, a significant relationship was found between the $CD14^+CD16^+$ monocyte ≤13·31% group and the $CD14^+CD16^+$ monocyte >13·31% group in respect of tumour size and pathological staging ($P < 0·05$). However, menstruation, lymph node metastasis, oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2) showed no such significant association with the level of $CD14^+CD16^+$ monocytes. In early-stage breast cancer patients, especially those with stage I and/or small tumour size (T1–T2), had high levels of $CD14^+CD16^+$ monocytes. The mean level of $CD14^+CD16^+$ monocytes was increased significantly in stage I patients (19·39 ± 9·4%) compared with stages II–IV patients (14·81 ± 5·0%) (Fig. 3). The higher level of $CD14^+CD16^+$ monocytes in early-stage patients suggests that the expanded $CD14^+CD16^+$ monocytes may be related to tumorigenesis of breast cancer.

The frequency of $CD14^+CD16^+$ monocytes were increased by MCF-CM

To evaluate the potential reason for the expansion in $CD14^+CD16^+$ monocytes in breast cancer patients, we

Table 1. Clinicopathological characteristics of the patients.

	$CD14^+CD16^+$ monocytes (%) ≤ 13·31% ($n = 32$)	$CD14^+CD16^+$ monocytes (%) > 13·31% ($n = 64$)	*P*-value
Age (years)			
≤Median (48 years)	12 (25)	36 (75)	0·083
>Median	20 (41·7)	28 (58·3)	
Menstruation			
Pre-menopause	14 (26·9)	38 (73·1)	0·148
Post-menopause	18 (40·9)	26 (59·1)	
Tumour size			
pT1	12 (23·1)	40 (76·9)	0·020
pT2–pT3	20 (45·5)	24 (54·5)	
Lymph node metastasis			
pN0	20 (30·3)	46 (69·7)	0·350
pN1–pN2	12 (40)	18 (60)	
pstage			
I	10 (22·7)	34 (77·3)	0·043
II–IV	22 (42·3)	30 (57·7)	
Hormone			
ER^-	12 (30)	28 (70)	0·558
ER^{+-+++}	20 (35·7)	36 (64·3)	
PR^-	16 (30·8)	36 (69·2)	0·562
PR^{+-+++}	16 (36·4)	28 (63·6)	
$HER2^-$	20 (37·0)	34 (63·0)	0·383
$HER2^{+-+++}$	12 (28·6)	30 (71·4)	

Data given as number (%). ER: oestrogen receptor; HER: human epidermal growth factor; PR: progesterone receptor.

cultured human PBMC-purified $CD14^+$ monocytes in complete RPMI-1640 with, and separately without, 25%, 50% or 75% MCF-CM for 24 h. The flow cytometry analyses results showed that, compared with the group that was treated with normal medium, the proportion of $CD14^+CD16^+$ monocytes was increased by almost three times in the presence of 25% MCF-CM and by almost five times in 50% and 75% MCF-CM (Fig. 4). These results demonstrate that MCF-CM can expand significantly the level of $CD14^+CD16^+$ monocytes.

$CD14^+CD16^+$ monocytes can be expanded by MCP-1

Several cytokines have been reported to induce the expansion of $CD14^+CD16^+$ monocytes *in vitro* and *in vivo*, including IL-10, macrophage colony-stimulating factor (M-CSF) and P-selectin [20–22]. In this study, we investigated which cytokines would be able to expand $CD14^+CD16^+$ monocytes in patients with breast cancer. Because the cytokines of TNF-α, IL-2, OPN and MCP-1

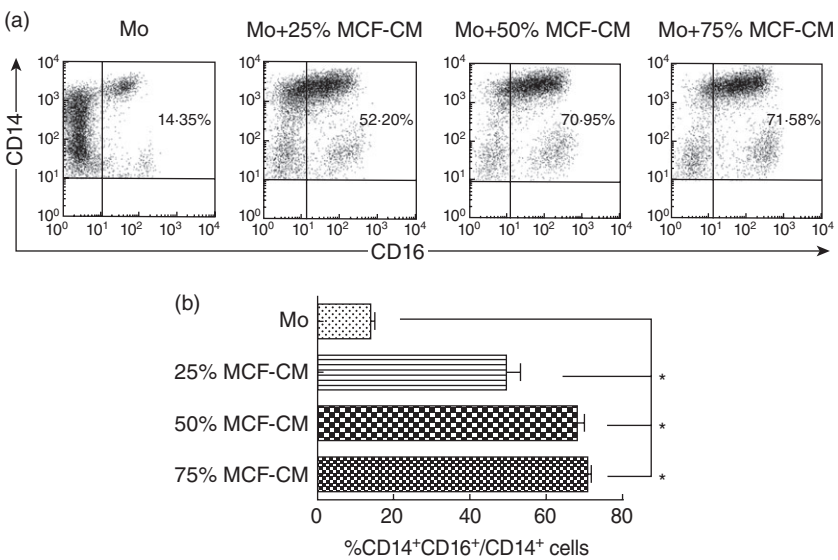

Fig. 4. $CD14^+$ monocytes were treated with normal medium, 25%, 50% and 75% Michigan Cancer Foundation-conditioned medium (MCF-CM) for 24 h. Cells were harvested and the expression of CD14 and CD16 were analysed by flow cytometry. (a) Flow cytometric density plots showed the $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes subsets after the monocytes were cultivated in the presence or absence of 25%, 50% and 75% MCF-CM for 24 h. The values in the density plots are the percentages of $CD14^+CD16^+$ monocytes. (b) Summary analyses of the percentages of $CD14^+CD16^+$ monocytes in the presence of 25%, 50% and 75% MCF-CM. Data are given as mean ± standard deviation (n = 3). $^*P < 0·05$.

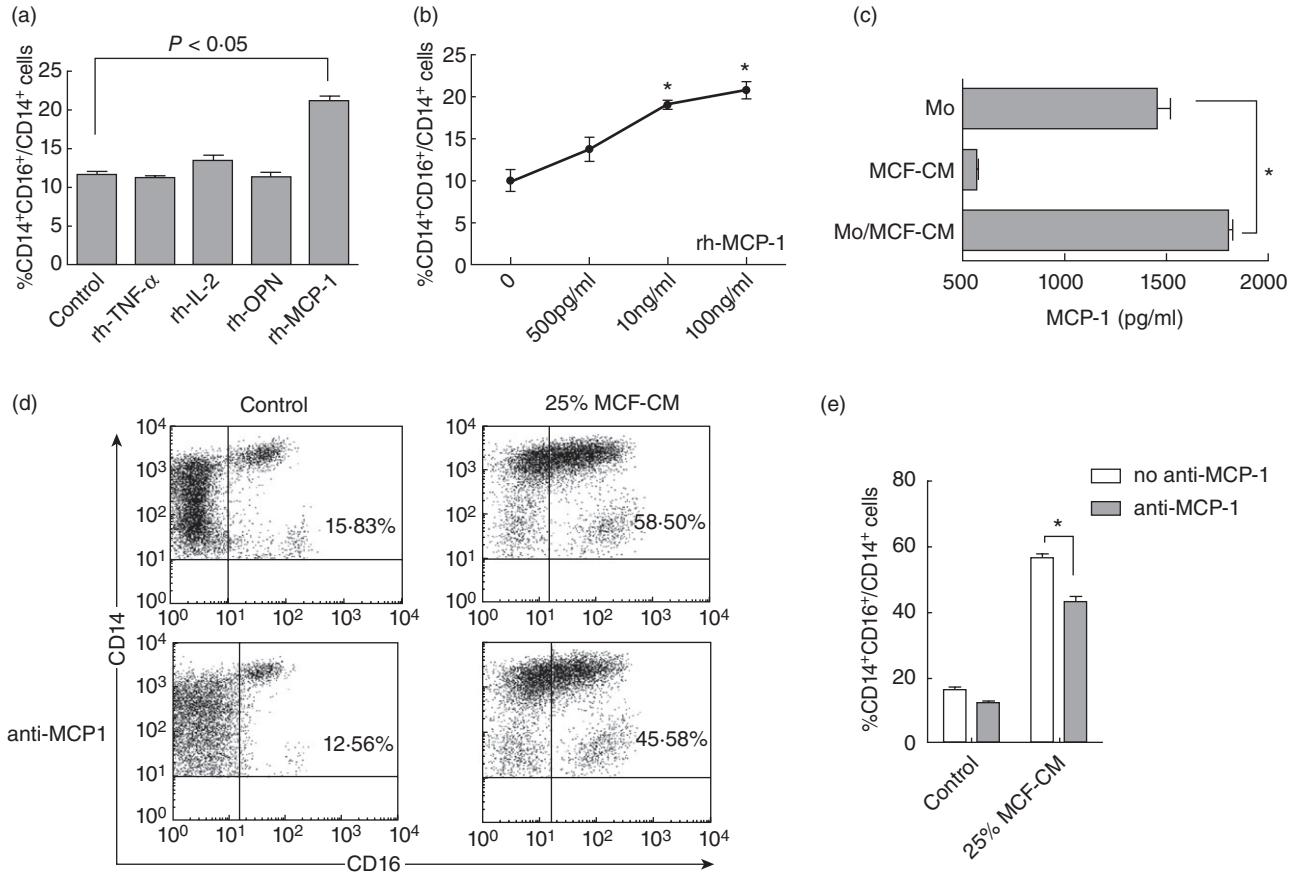

Fig. 5. (a) Monocytes (5×10^5/ml) were stimulated with recombinant human-tumour necrosis factor (rh-TNF)-α (50 ng/ml), rh-interleukin (rh-IL)-2 (100 units/ml), rh-osteopontin (OPN) (200 ng/ml), rh-monocyte chemotactic protein 1 (MCP-1) (100 ng/ml), respectively, or with only the medium as control for 24 h. The monocytes were then harvested and the expression of CD14 and CD16 was analysed by flow cytometry. (b) Monocytes were cultivated in the presence or absence of different concentrations of rh-MCP-1 (0·5 ng/ml, 10 ng/ml, 100 ng/ml) for 24 h. (c) Monocytes (5×10^5/ml) were co-cultured with 25% Michigan Cancer Foundation-conditioned medium (MCF-CM). After 24 h, the amount of MCP-1 in the culture supernatant was determined by enzyme-linked immunosorbent assay. (d) The flow cytometric density plots show the $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes subsets after monocytes were cultivated in the presence or absence of 25% MCF-CM (upper column) or anti-MCP-1 monoclonal antibodies (mAb) (5 ug/ml) (lower column) for 24 h. The values in the density plots are the percentages of $CD14^+CD16^+$ monocytes. (e) The summary of analyses of the percentages of $CD14^+CD16^+$ monocytes in the presence of 25% MCF-CM or anti-MCP-1 mAb. All the data are expressed as means ± standard deviation of three independent experiments. $^*P < 0{\cdot}05$.

were involved in anti-tumour immunity or tumour tolerance, we stimulated monocytes with these cytokines and detected the phenotypic change of the monocytes. As shown in Fig. 5a, the frequency of $CD14^+CD16^+$ monocytes was increased significantly when stimulated by 100 ng/ml rh-MCP-1 ($P < 0{\cdot}05$), not by other cytokines TNF-α, IL-2 and OPN. Moreover, as shown in Fig. 5b, the effect of MCP-1 on the frequency of $CD14^+CD16^+$ monocytes was dose-dependent.

We then detected the level of MCP-1 in the supernatant of monocytes, MCF-CM and in the co-culture supernatant of monocytes and MCF-CM by ELISA. As shown in Fig. 5c, MCP-1 in monocytes treated with 25% MCF-CM for 24 h was raised compared with control ($P < 0{\cdot}05$). The anti-MCP-1 mAb can partly inhibit the increased $CD14^+CD16^+$ monocyte frequency by MCF-CM (56·43% *versus* 43·09%, $P < 0{\cdot}05$) (Fig. 5d and e). These data suggest that MCF-CM can stimulate monocytes to secret more MCP-1, which may be one of the reasons why $CD14^+CD16^+$ monocytes increased in patients with breast cancer.

Discussion

Human peripheral blood monocytes are a heterogeneous population and are divided into two subsets based on the expression of CD16. In this study, we initially described the heterogeneity of monocytes in the peripheral blood of patients with breast cancer by flow cytometry. We found that the $CD14^+CD16^+$ monocyte subpopulation can be induced and expanded in breast cancer patients. This result is consistent with the previous report that $CD14^+CD16^+$ monocytes were expanded spontaneously in patients

with metastatic gastrointestinal carcinoma [23]. More importantly, we showed for the first time that the levels of $CD14^+CD16^+$ monocytes were significantly negatively associated with tumour size and staging. These results demonstrate that dynamic changes occur in two distinct monocyte subset levels in breast cancer patients, and that stochastic profiling of this monocyte/macrophage system may hold clinical utility with respect to the diagnosis of breast cancer.

In patients with metastatic melanoma elevated monocyte counts are associated with poor survival [24], suggesting a role for monocytes in the development of cancer. The concrete roles of two distinct monocyte subsets in breast cancer remain unclear. Several studies have demonstrated that the $CD14^+CD16^+$ and $CD14^+CD16^-$ monocyte subsets show functional differences in migration, cytokine production and differentiation into macrophages or dendritic cells [25–28]. The homologue population of $CD14^+CD16^+$ mouse monocytes migrates preferentially to the salivary glands, and develops into DCs *in vivo* [29,30]. The DCs that are generated from $CD14^+CD16^+$ monocytes can induce $CD4^+$ T cells preferentially to Th2 polarization, and these DCs may represent a subset of regulatory DCs which can induce tumour immune tolerance [9,10]. There are also studies indicating that $CD14^+CD16^+$ monocytes are involved in the tumour immune response [31]. We found that a considerable proportion of $CD14^+CD16^+$ monocytes exist in tumour tissues, so we hypothesized that $CD14^+CD16^+$ monocytes could migrate from peripheral blood into the tumour site and then differentiate into regulatory DCs, which could be involved in the immune tolerance of tumour cells.

Schlitt *et al.* [12] reported that the increased number of $CD14^+CD16^+$ monocytes is associated with coronary atherosclerosis. Also, another clinical study [32] has indicated that the peak levels of $CD14^+CD16^-$ monocytes, but not those of $CD14^+CD16^+$ monocytes, are significantly negatively associated with the extent of myocardial salvage in patients with acute myocardial infarction (AMI). $CD14^+CD16^+$ monocytes also correlate with disease progression in chronic HIV-infected patients [33]. Importantly, $CD14^+CD16^+$ monocytes have the potential role to serve as an osteoclast precursor marker in inflammatory arthritis [34]. Therefore, we evaluated the role of $CD14^+CD16^+$ monocytes in diagnosing breast cancer and analysed the correlation between the levels of $CD14^+CD16^+$ monocytes and breast cancer patients' clinicopathological parameters. In the present study, ROC curves were used to evaluate the performance of $CD14^+CD16^+$ monocytes in diagnosing breast cancer, but the data demonstrated that the sensitivity (68·7%) and the specificity (79·6%) were not very high. Therefore, measuring the $CD14^+CD16^+$ monocyte subset should be used in combination with other factors. Furthermore, $CD14^+CD16^+$ monocytes were significantly negatively correlated with tumour size and TNM staging in breast cancer patients. Early-stage (pstage I) patients have higher rates of $CD14^+CD16^+$ monocytes. Overall, these results suggest that $CD14^+CD16^+$ monocytes play a critical role in tumorigenesis.

It was reported recently that many cytokines, such as IL-10 and M-CSF, may increase the expression of CD16 in monocytes [22,23,35–37]. However, it is not clear which cytokines can regulate the $CD14^+CD16^+$ monocytes in breast cancer patients. Our data from experiments *in vitro* show that MCF-CM can stimulate monocytes to secrete more MCP-1, which can increase $CD14^+CD16^+$ monocytes, whereas anti-MCP-1 can inhibit the increase of $CD14^+CD16^+$ monocytes by MCF-CM. Interestingly, $CD14^+CD16^+$ monocytes cannot be restored fully to the level of the control group of monocytes which do not stimulate with MCF-CM. This suggests that there are also other cytokines to regulate monocyte heterogeneity in the tumour microenvironment. The chemokine MCP-1 has been shown to be expressed minimally in normal breast epithelial duct cells, but is expressed extensively in breast tumour cells. The expression of MCP-1 is associated strongly with the progression of breast cancer and with the advanced disease course [17], but the tumour microenvironment is extremely complex *in vivo* in cancer patients. There should be many other cytokines take part in the regulation of monocytes heterogeneity together with MCP-1 in the different stage and grade of cancer which needs further investigation.

Previously, it has been demonstrated that $CD14^+CD16^+$ monocytes express high levels of CX3CR1 and low levels of CD62L and CCR2, the receptor for MCP-1. By contrast, $CD14^+CD16^-$ monocytes express high levels of CCR2 [38]. However, our study found that MCP-1 can increase $CD14^+CD16^+$ monocytes. Significantly, this provides very strong evidence that the role of MCP-1 in $CD14^+CD16^+$ monocytes is not dependent on the CCR2 receptor. Future research should focus on the mechanisms by which MCP-1 increases $CD14^+CD16^+$ monocytes.

In summary, our results show that breast cancer patients have an increased frequency of $CD14^+CD16^+$ monocytes which are related to tumour size and tumour stage. Furthermore, it has been found that MCF-CM stimulates monocytes to excrete more MCP-1, which is involved in augmenting $CD14^+CD16^+$ monocytes. Our study provides further and substantial evidence that $CD14^+CD16^+$ monocytes should be considered as a useful indicator for the early diagnosis of breast cancer.

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Disclosure

None.

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